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The *myc* oncogenes encode nuclear phosphoproteins that function as transcription factors in mammalian cells. To molecularly dissect regions of potential regulation and possible protein-protein interaction, site-directed mutagenesis of the amino terminal transcription activation domain (TAD) of the avian v-Myc oncoprotein was performed. C3H10T1/2 fibroblasts were transiently transfected with GAL4 fusion constructs expressing selected regions of the v-Myc TAD and tested for their ability to activate transcription of a reporter plasmid containing recognized GAL4 protein binding sites. The results show a significant increase in transcription activation by v-Myc proteins deleted for a highly conserved domain of the Myc TAD referred to as Myc Homology Region II (MHR II). This could implicate MHR II as a region of negative regulation in v-Myc. A second interesting observation centers on the extreme 3' end of the v-Myc TAD which appears to negatively regulate the ability of all constructs to activate transcription. These results suggest that separate areas of the v-Myc amino terminal TAD have distinct roles in regulating the function of Myc as a transcription factor. By using another chimeric protein consisting of the HSV VP16 TAD and the carboxy terminus of v-Myc, Myc's role in transformation was assessed. It has been demonstrated that the amino terminus of v-Myc performs a specific function in cellular transformation that cannot be replaced by a heterologous TAD.

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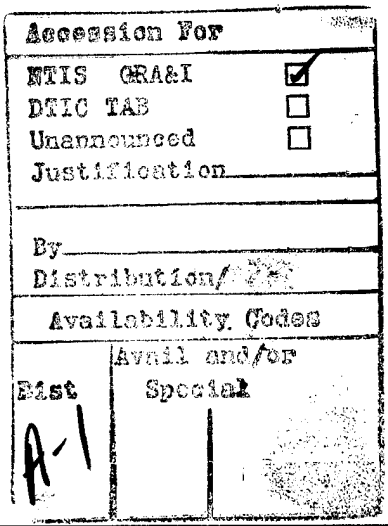

Elizabeth J. Japaroutsky 6/7/95
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INTRODUCTION

The *myc* oncogene was first discovered as the transforming sequence present in the avian leukemia retrovirus MC29, which is found to be the cause of myelocytomatosis and other tumor types in infected birds (Sheiness *et al.*, 1979; Graf & Beug, 1978). The v-*myc* cellular homolog, c-*myc*, as well as other members of the *myc* gene family (N-*myc*, L-*myc*, B-*myc*, S-*myc*) are implicated in a wide variety of human cancers, for example lymphomas, neuroblastomas, and carcinomas. Chromosomal rearrangements, mutation, and amplification of the *myc* genes result in increased expression levels of the Myc oncoprotein and contribute to the development and progression of human malignancies (Marcu *et al.*, 1992). Of particular interest is the relationship between c-*myc* amplification and human breast cancer. Berns *et al.* (1992) and Escot *et al.* (1986) have demonstrated that up to 20%-30% of breast cancers contain amplifications of the c-*myc* gene. Additionally, studies using antisense oligonucleotides to inhibit the expression of the c-*myc* protein in an estrogen-dependent human breast cancer cell line (MCF-7) result in growth arrest of these cells, implicating *myc* as a crucial element in breast cancer cell growth (Watson *et al.*, 1991). Taken together, these observations suggest that the Myc oncoprotein performs a pivotal rloe in a variety of human cancers.

My research interests are focused on the protein encoded by the retroviral v-*myc* gene. Specifically, I am examining the v-Myc amino terminus to identify regions that are important for the regulation of this oncoprotein. Previous work in our lab has generated a general functional map of v-Myc. Areas of the protein responsible for the nuclear localization, interaction with Myc's protein binding partner, Max, and the ability of Myc:Max heterodimers to bind specifically to a hexamer DNA sequence referred to as an E-box are localized to the carboxy terminus of v-Myc (Min *et al.*, 1993). The amino terminus of v-Myc encodes Myc's ability to activate transcription; in particular, two subregions of the amino terminus, residues -32--42 (in which -32 refers to 32 amino acids encoded by the MC29 *gag* gene) and residues 90--219, are each able to independently transactivate a reporter gene, indicating that the v-Myc amino terminus is capable of being separated into two funcional transcription activation domains (TADs) (Min & Taparowsky, 1992). Finally, the ability of v-Myc to cause cellullar transformation is encoded by two distinct regions of the protein. Both the DNA binding domain (basic region) and the region responsible for heterodimerization with Max (helix-loop-helix/leucine zipper region) of the v-Myc carboxy terminus are necessary for cellular transformation. In addition, the first 137 amino acids of the amino terminus of v-Myc are required in order for v-Myc to transform cells (Min & Taparowsky, 1992). This points to an important role for the amino terminus of v-Myc, as this region is necessary for both transcriptional activation and cellular transformation. Therefore, a detailed analysis of this

crucial region of v-Myc is important to begin to identify and recognize possible areas of regulation of this oncoprotein.

An important consideration of the above observations is that the Myc amino terminus is required for both transcriptional activation and cellular transformation. An obvious prediction would be that the Myc protein, either v-Myc or c-Myc, would activate transcription of target genes that would be important to bring about a transformed phenotype. However, recent studies have indicated that perhaps these two activities of Myc are not necessarily linked. First, a study by Li *et al.* (1994) has shown that a highly conserved region among Myc family members [MHR II (Figure 1, see Appendix)] can be deleted from the c-Myc protein without affecting c-Myc's transactivation potential; however, the same c-Myc mutant is severely compromised in its ability to transform cells. Next, inhibition of transactivation of a reporter gene is seen when two c-Myc N-terminal phosphorylation sites (Thr-58 and Ser-62) are mutated. However, this altered Myc protein still efficiently transforms fibroblasts (Gupta *et al.*, 1993). Studies such as these suggest that the ability of Myc to activate transcription can be separated from its ability to transform cells, an important realization that could help identify ways to address Myc's role in human malignancies.

A possible mechanism by which the v-Myc amino terminus is able to perform the varied functions of both transactivation and transformation is by interacting with proteins necessary for activation of transcription and/or cellular transformation. Experiments implicating protein-protein interactions involving the Myc amino terminus are based on the phenomenon known as squelching. Theoretically, an activation domain could interact with its target protein whether or not the activator is bound to DNA. When the activator is bound to DNA, interaction with an appropriate target protein (coactivator or basal transcription factor) will result in transcriptional activation. However, if this target is in limited quantities, overexpression of an activator could result in interactions when not bound to DNA. This would repress transcription by making the target unavailable for interaction with the activator (Ptashne, 1988). Resar *et al.* (1993) demonstrated this phenomenon using B-Myc, a Myc family member that consists of 168 amino acids that are homologous to the c-Myc transcription activation domain but which lacks a basic helix-loop-helix/leucine zipper motif. In these experiments, full length B-Myc was transiently transfected into cells along with the fusion protein GAL4 c-Myc (1-262), which is a strong activator. B-Myc was able to squelch the transcriptional activity of GAL4 c-Myc (1-262), resulting in a greater than 50% reduction in reporter gene expression. A possible explanation of this observation is B-Myc sequesters a unique coactivator necessary for the function of the Myc proteins. Min *et al.* (1994) have demonstrated a similar phenomenon with the v-Myc TAD. The full length TAD (residues -32--244) and subregions -32--42 and 90--219 were expressed as LexA-v-Myc fusion proteins and

used to squelch the full length v-Myc TAD expressed as a GAL4 fusion protein. All three LexA-v-Myc fusions were able to reduce v-Myc transactivation by at least 50%. These results indicate that the amino terminus of v-Myc does indeed participate in protein-protein interactions, and that these interactions could possibly be vital to the overall regulation of the functions of this oncoprotein. This further supports the need to critically examine the Myc amino terminus.

The above observations indicate the complexity associated with the functions of the v-Myc protein in cells. Despite the continual flood of new information regarding the Myc oncoprotein that is acquired everyday, much remains to be discovered. It is the goal of my research to examine possible regulatory regions of the v-Myc amino terminus with the ultimate objective of using this information to identify interacting proteins that could lead to the transformed phenotype seen in malignancies. To carry out this goal, mutagenesis of the v-Myc amino terminus has initially been performed to investigate possible regulatory regions important in transactivation. Mutagenesis consisted of generating deletions within the v-Myc TAD that allow for a thorough analysis of the transactivation potential of the entire amino terminus. Additionally, the role of the v-Myc amino terminus, which contains the v-Myc TAD, in transactivation versus transformation studies has been performed using chimeric proteins, detailed in the next section. The results of the experiments performed to date provide a good basis for further study of this oncoprotein.

BODY

To begin characterizing the functional regions of the v-Myc transcription activation domain, mutagenesis of the v-Myc amino terminus was performed. Initially, a series of 5' and 3' deletions of the v-Myc TAD were generated. The 3' deletions were created by restricting a plasmid called GAL4/v-Myc (-32--244) (Min & Taparowsky, 1992) containing v-Myc residues -32--244 fused 3' to the DNA binding domain (residues 1-147) of the yeast transcription factor GAL4. The Sal I/Kpn I linearized GAL4/v-Myc (-32--244) plasmid was then digested with Exonuclease III and S 1 nuclease (Erase-A-Base Kit, Promega) to create 3' deletions of various sizes. Before each of these deletions was ligated and used to transform *E. coli,* the 3' ends were modified with the addition of a Bgl II linker (unique to the v-Myc oncoprotein). The reason for addition of the Bgl II linkers was twofold: first, this unique restriction site will aid in subcloning selected v-Myc deletions into other expression vectors, and second, the use of Bgl II linkers in the 5' and 3' deletions will make it possible to combine 5' and 3' mutations to create internal deletions or duplications.

The 5' deletions of the v-Myc TAD were generated in a similar manner. v-Myc residues -20--244 had previously been cloned into the pBluescript vector (M. Dorsey and E. Taparowsky, unpub. results). This construct was restricted with Kpn I and Hind III to linearize the plasmid, and the Promega Erase-A-Base system was once again utilized to generate deletions in the v-Myc TAD, this time starting from the 5' end at amino acid -20. Bgl II linkers were ligated to the 5' ends of each v-Myc deletion in order to restore the correct reading frame of the v-Myc sequence (if necessary) and to allow for further subcloning. Because these 5' v-Myc deletions were created in a pBluescript vector, each was subcloned into the GAL4 (1-147) vector for use in mammalian cell transcription assays. The GAL4 (1-147) vector contains a modified polylinker in which the BamH I restriction site has been destroyed and a Bgl II site has been created (Min & Taparowsky, 1992). When translated, all the Myc deletions will be expressed as chimeric proteins between the yeast GAL4 DNA binding domain and varying lengths of the v-Myc transcription activation domain. Constructs were ligated and used to transform *E. coli* strain DH5. All the deletions were sequenced by the dideoxy chain termination method using the Sequenase 2.0 kit (USB). Sequencing the constructs determined the extent of the deletion and, for the 5' deletions, also confirmed the correct reading frame of v-*myc*. Twenty-two clones harboring deletions in the v-Myc TAD have been created using this procedure: 14 are 3' deletions and 8 are in-frame 5' deletions (Figure 2, see Appendix).

Analysis of the transcriptional activity of each of these v-Myc mutants was assayed in the mouse fibroblast cell line C3H10T1/2, essentially as described by Min & Taparowsky

(1992). C3H10T1/2 cells were transiently transfected by the calcium phosphate precipitation method. Precipitates used to transfect the cells contained 5 μg of test activator plasmid, 5 μg of a chloramphenicol acetyl transferase (CAT) reporter plasmid, and 5 μg of the β–galactosidase expression vector, RSV-LacZ (Jaynes et al., 1986). The reporter construct used in these assays consists of five GAL4 protein binding sites, a minimal promoter (E1B TATA), and the CAT reporter gene {$(GAL4)_5$ E1B TATA CAT}. Following transfection and growth in complete media for 40-50 hr., cells were harvested, cell extracts were prepared, and transfection efficiency was estimated by measuring β-galactosidase activity. Cell extracts were normalized using β-galactosidase activity as a standard and equivalent amounts of extract assayed for the expression of the CAT protein. The percent conversion of the non-acetylated form of chloramphenicol to the acetylated form was calculated by liquid scintillation counting and reflects the strength of each GAL4 v-Myc activator protein. The positive control used in these assays is the full length v-Myc TAD (residues -32--219) expressed as a fusion protein with the GAL4 DNA binding domain {GAL4 v-Myc (-32--219)}. The negative control used was the GAL4 (1-147) vector containing no v-*myc* sequences. The relative CAT activity of all constructs tested was expressed as a percentage of the positive control, which was set at 100%.

As can be seen from Figure 3 (see Appendix), substantial CAT activity (at least wild-type or greater) is observed among the smallest v-Myc fragments tested. Constructs consisting of v-Myc residues -32--27, -32--97, -32--98, and -32--112 activate CAT expression as well as the positive control, GAL4 v-Myc (-32--219). One observation made is that all of these potent activators are deleted for **M**yc **H**omology **R**egion **II** (MHR II, Figure 1), residues 114 through 130. The first 3' deletion that contains MHR II is -32--134, and this construct is only 50% as efficient as the full length v-Myc TAD in activating transcription. These results could implicate the conserved region of MHR II as a negative regulatory domain.

Another interesting observation is the difference in relative CAT activity between v-Myc constructs -32--12 and -32--27. It appears that the intervening 15 amino acids between v-Myc residues 12 and 27 are necessary for the transcriptional activation properties measured using these 3' deletions. Motifs common in the transcription activation domains of described eukaryotic transciption factors are rich in acidic amino acids, glutamine, or proline (Mitchell & Tjian, 1989). The amino acid sequence of the first 12 residues of v-Myc show no such "motif," whereas there are 5 acidic residues, 1 glutamine, and 1 proline found in the next 15 amino acids of v-Myc. These residues could be important for the transactivation potential of the v-Myc (-32--27) clone.

Lastly, these 3' deletions serve to further restrict the N-terminal activation domain of v-Myc from the identified region of -32--42 (Min & Taparowsky, 1992) to -32--27. These minimal residues (v-Myc -32--27) are sufficient to activate the CAT reporter gene above that of

the wild-type activation domain.

The 5' v-Myc deletions were tested in the same manner as the 3' deletion constructs. Figure 4 (see Appendix) shows the results obtained when the 5' v-Myc deletions were assayed for their ability to activate transcription of the CAT reporter gene. As seen in Figure 4, all of the constructs exhibited less than 50% of the activity of the full length v-Myc TAD construct {GAL4 v-Myc (-32--219)}. This was unexpected based upon the results of Min & Taparowsky (1992), which demonstrated that a C-terminal region of the v-Myc TAD, specifically GAL4/v-Myc (90--219), was an extremely strong activator, exhibiting a transactivation potential at least three-fold higher than that of the full length v-Myc TAD. All of the 5' v-Myc deletions, except GAL4 v-Myc (107-219), contain this activating region (residues 90--219). Due to this discrepancy and because the positive control being used in all experiments only extended to amino acid 219 of v-Myc {GAL4 v-Myc (-32--219)}, each of the v-Myc 5' deletions was modified further to remove residues 220-244. This was acconplished by restricting each of the GAL4 5' v-Myc deletion constructs with Sal I, which cuts at v-Myc residue 219 and at a site within the vector polylinker. These clones were then religated and resequenced. DNA sequencing confirmed that modification of the 5' clones retained an in-frame stop codon within the polylinker.

These new v-Myc clones were used to transiently transfect C3H10T1/2 cells, and CAT activity was measured. Figure 5 (see Appendix) displays these results. Except for GAL4 v-Myc (107-219), each of the new 5' clones exhibited increased transcriptional activity when compared with its longer 5' counterpart. Examination of the sequence between v-Myc residues 220 and 244 reveals two interesting regions. The first of these is a casein kinase II (CK II) phosphorylation site (Luscher et al., 1989). The other is an acidic region stretching from residues 230 to 238, composed of 8 acidic amino acids out of 9 residues total. Based on these observations, this C-terminal area of the v-Myc TAD may also represent a negative regulatory domain.

Finally, to further examine the transforming properties of the v-Myc oncoprotein, another chimeric protein was constructed utilizing a portion of the transcription activation domain of the Herpes simplex virus protein 16 (VP16). A vector (pNLVP16) containing residues 411-455 of VP16 (which can serve as a transcription activation domain) driven by the SV40 promoter was obtained from Dr. C.V. Dang. The polylinker of this vector was restricted with Xho I and Xba I. GAL4/v-Myc (-32--416) was restricted with Sal I (site at residue 219 and complementary to Xho I) and Xba I (site located in polylinker). The v-Myc 219--416 fragment was ligated into pNLVP16. This VP16-v-Myc fusion was tested in two ways. First, the transcriptional activation function of this clone was assessed on a reporter plasmid carrying the natural Myc binding site. This reporter, $(MBS)_3$ TK CAT, carries three tandemly repeated

consensus Myc binding sites (-CACGTG-) upstream of the thymidine kinase promoter, which is linked to the chloramphenicol acetyl transferase (CAT) gene. The dimerization motif of v-Myc is present in this pNLVP16 v-Myc (219--416) clone, so binding to a consensus E-box was expected to occur. The VP16 TAD is also present in this fusion construct, so transcriptional activation was predicted to happen. As can be seen from Figure 6 (see Appendix), the pNLVP16 v-Myc clone was able to activate transcription from the E-box-containing reporter when C3H10T1/2 cells were transiently transfected with 5 μg reporter plasmid and 5 μg test activator plasmid (either pMC29 or pNLVP16 v-Myc (219--416)). The VP16 v-Myc fusion did not activate transcription as efficiently as the full length v-Myc construct (pMC29), however.

This fusion construct was also tested for its ability to cooperate with an activated H-*ras* gene to transform C3H10T1/2 cells. Stable transfections of C3H10T1/2 cells were performed. Separate calcium phosphate precipitates containing 200 ng pT24 H-*ras*, 600 ng pMC29, 600 ng pNLVP16 v-Myc (219--416), and combinations of 200 ng pT24 and 600 ng of each v-*myc* clone were made. Two 100mm plates of cells (approx. 1 x 10^6 cells total) were tested per group. Two hours prior to addition of the precipitates, the cells were treated with 10 μl 100 mM chloroquine. 5-6 hours post transfection the cells were refed complete medium (containing 10% fetal bovine serum). 24 hours after transfection, the cells were split 1:3 and maintained in medium with reduced serum (5% FBS). Cells were fed every 3-4 days. After 14 days, the cells were fixed with methanol, stained with Giemsa, and focus formation was assessed. The number of foci formed by the cooperation of pT24 H-*ras* and wild type pMC29 was considered the positive control, and this value was set at 100%. The amount of foci obtained with pT24 H-ras and pNLVP16 v-Myc (219--416) was calculated and compared with the positive control. As can be seen from Figure 7 (see Appendix), *ras* alone can transform C3H10T1/2 cells to approximately 47% of the value obtained when *ras* and full length v-Myc (pMC29) transform cells. However, *ras* and the VP16 v-Myc chimera do not cooperate to form foci, as can be seen from the value that is comparable to *ras* alone. These results indicate that it is not the mere presence of a transcription activation domain in Myc that is necessary to bring about cellular transformation. The v-Myc amino terminus apparently encodes a specific function that allows v-Myc to cooperate with *ras* to transform cells.

CONCLUSIONS

As can be seen in Figures 3 through 5, different regions of the v-Myc oncoprotein impart different activities on v-Myc's ability to function as a transcriptional activator. The determination of a possible inhibitory role for residues downstream of v-Myc amino acid 112 (Figure 3) is interesting due to the fact that this region contains one of the highly conserved "boxes" in the Myc family of proteins, MHR II (Figure1). To better address this initial observation, site-specific mutagenesis using PCR technology will be employed to examine residues critical to the observed negative function of MHR II. Interestingly, this conserved region of the c-Myc protein has been shown to be essential for cellular transformation, indicated by the fact that deletion of MHR II from c-Myc or alteration of a conserved tryptophan residue to either glycine or glutamic acid results in a dramatic decrease in Myc's ability to cooperate with *ras* to transform cells (Brough *et al.*, 1995). Also, Myc's role as a repressor of transcription through initiator elements is determined in part by MHR II. Repression of transcription from initiator elements in the promoters of two genes, C/EBPα and albumin is dependent on an intact MHR II domain in the c-Myc protein (Li *et al.*, 1994). The role of this region of v-Myc in transcriptional activation and later in cellular transformation will be assessed by my studies.

Comparison of Figures 4 and 5 indicate that residues 220 through 244 of v-Myc could also bestow a negative regulatory function on v-Myc. Within this region lies an *in vivo* CK II phosphorylation site (Luscher *et al.*, 1989) and an extremely acidic region adjacent to the CK II site. The function of this acidic region is unknown. I therefore propose to use PCR-mediated mutagenesis to alter these two domains to determine if the negative function is lost. The CK II site will be altered from threonine and serine residues to small, non-charged residues like glycine and also to charged acidic residues that would mimick the outcome of phosphorylation. The acidic region will also be altered to residues that are not charged to reduce the overall negative charge of this region. Both of these domains (CK II site and acidic region) can also be deleted to assess what effect removal of these areas has on overall v-Myc amino terminal function.

Lastly, Figures 6 and 7 provide solid evidence that the v-Myc amino terminus does indeed perform a special function in cellular transformation. The pNLVP16 v-Myc (219--416) construct retains the DNA binding and dimerization motif of v-Myc and contains an activation domain (provided by VP16). It is therefore able to activate transcription from a reporter containing Myc protein binding sites (Figure 6). However, this VP16 TAD cannot replace the v-Myc TAD in transformation studies. This indicates that v-Myc does not simply bind to target genes and activate transcription to bring about cellular transformation. On the contrary, it is

hypothesized that Myc participates in other activities (for instance, protein-protein interactions) that help to bring about the transformed phenotype. This experiment serves as a good basis to begin searching for probable v-Myc TAD-interacting proteins.

Analysis of the amino terminus of the v-Myc oncoprotein should help us to gain a better understanding of two important functions of this region: transcriptional activation and cellular transformation. An ultimate goal of these analyses is to gain information that could be used to understand and perhaps eventually counteract the harmful effects associated with Myc protein overexpression in various human malignancies.

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APPENDIX

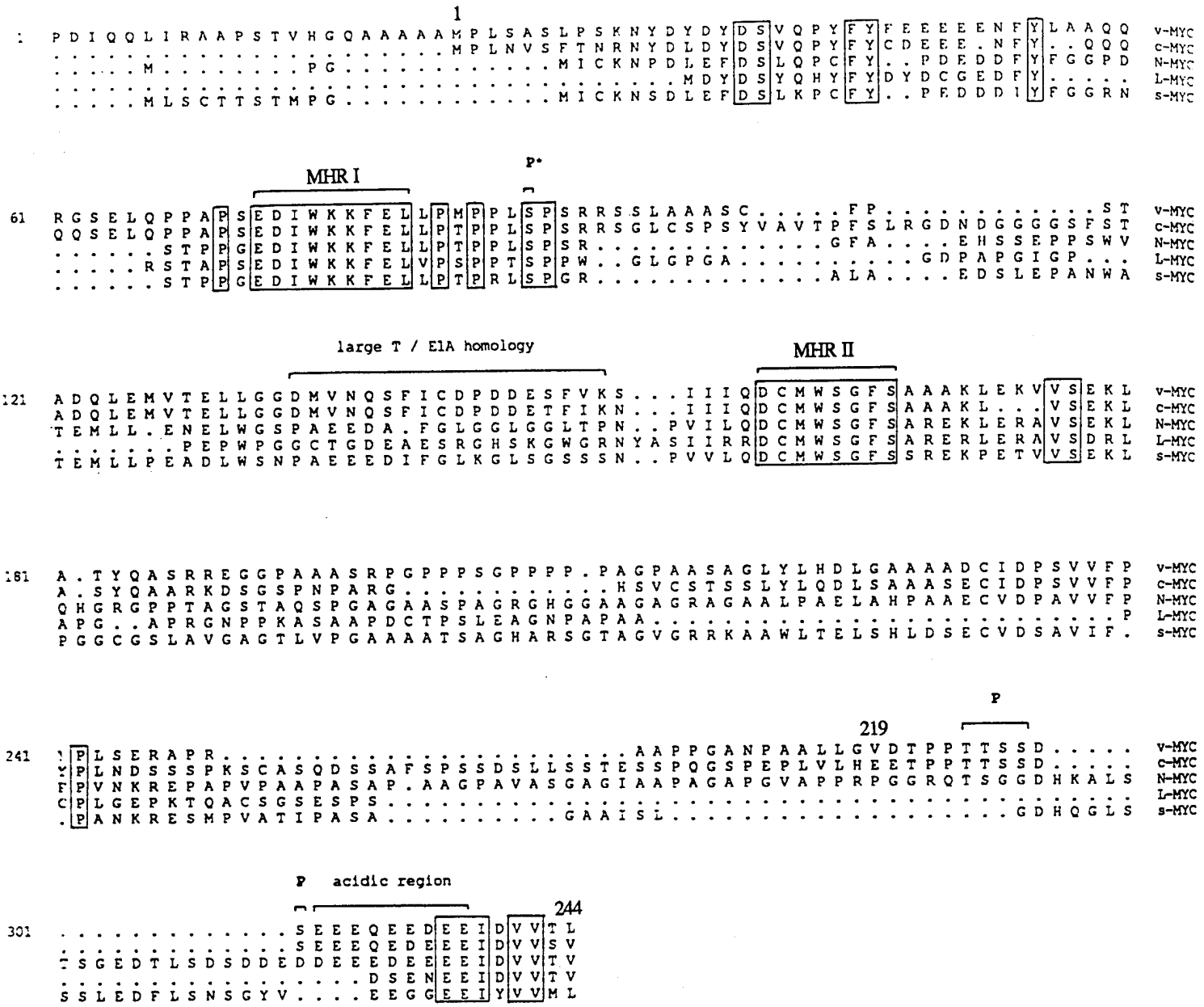
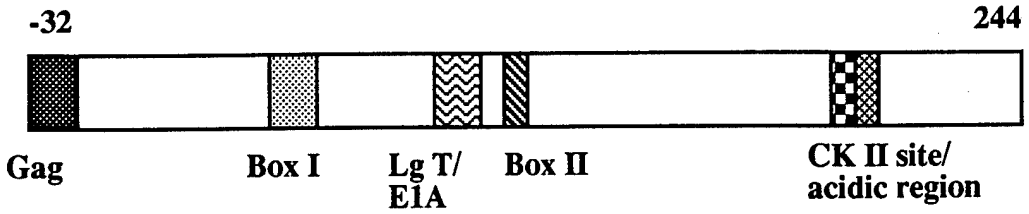
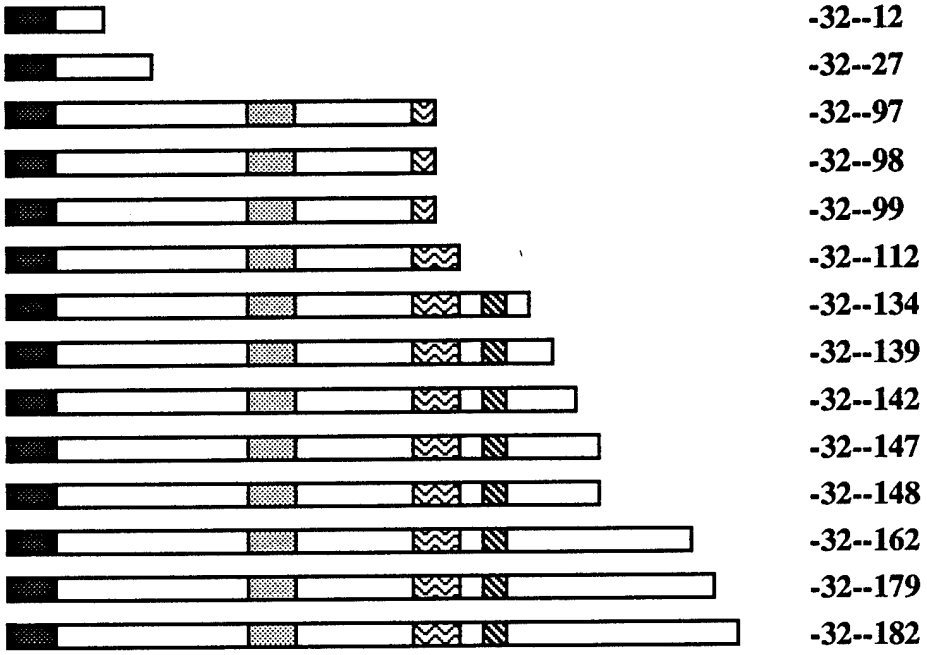


Figure 1. Amino acid sequences of the amino termini of the Myc family of oncoproteins. Conserved domains are boxed. Sequence adapted from Meichle *et al.* (1992).

AMINO TERMINUS OF V-MYC



3' Deletions of v-Myc TAD



5' Deletions of v-Myc TAD

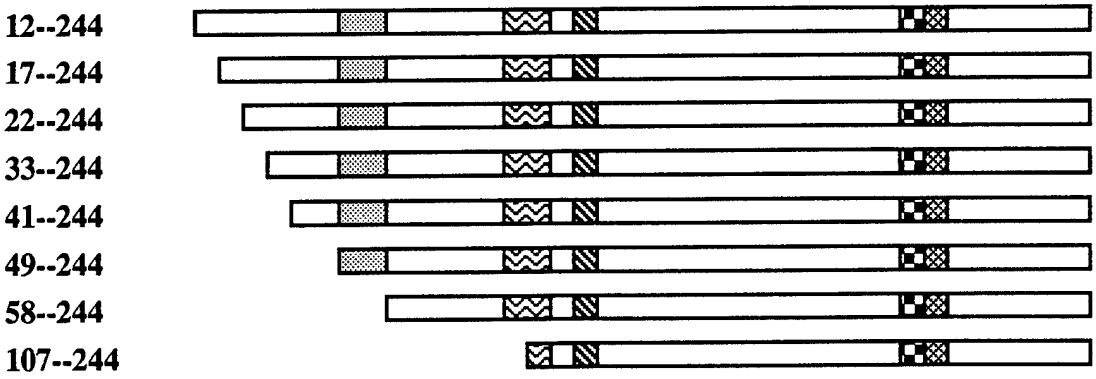


Figure 2. Diagramatic representations of deletions in the v-Myc oncoprotein.

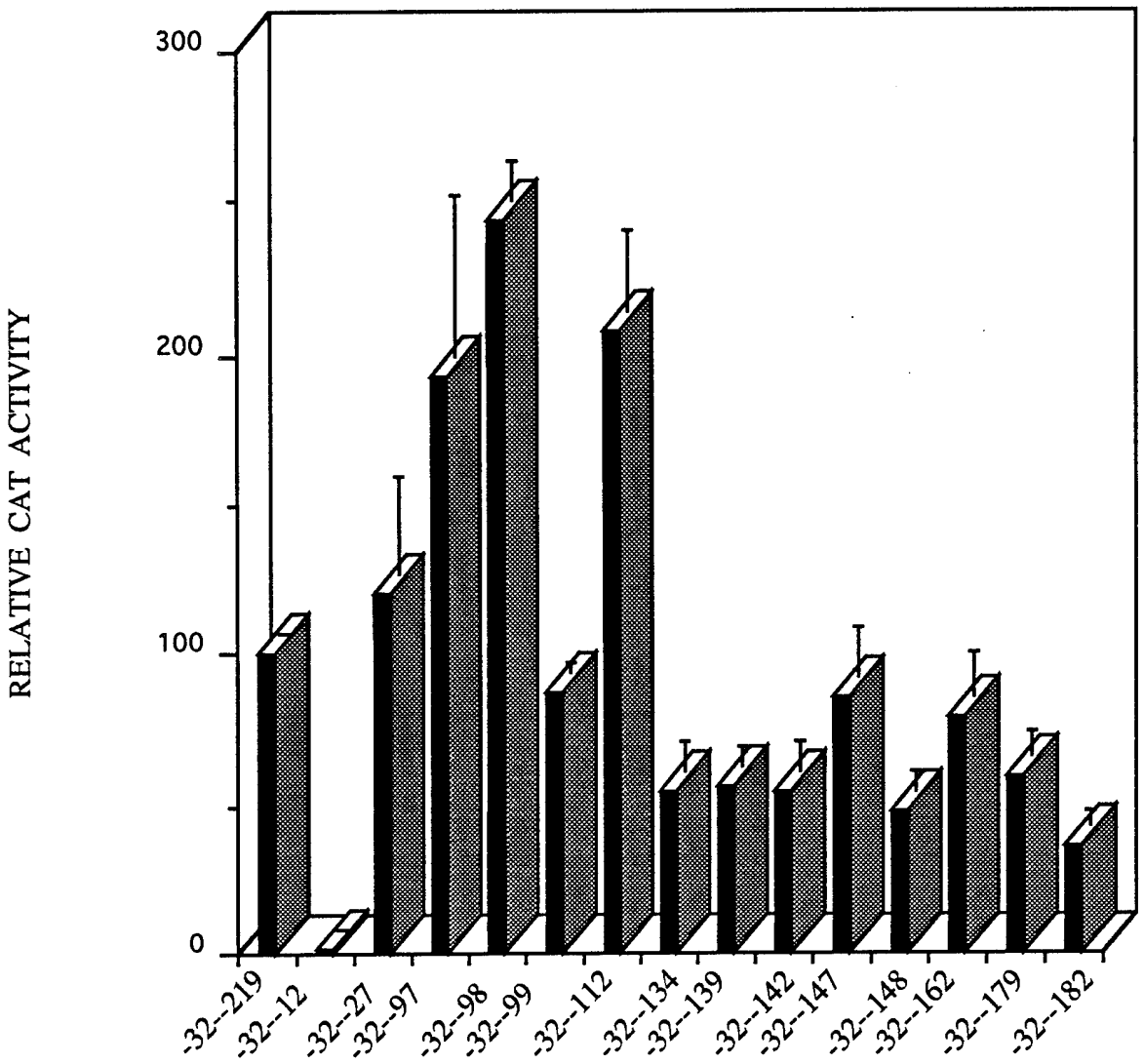


Figure 3. Transcriptional activation properties of the GAL4-v-Myc 3' deletion mutations. Relative CAT activity is presented as a percentage of the positive control, GAL4 v-Myc (-32--219). Each value represents the average of at least four independent measurements.

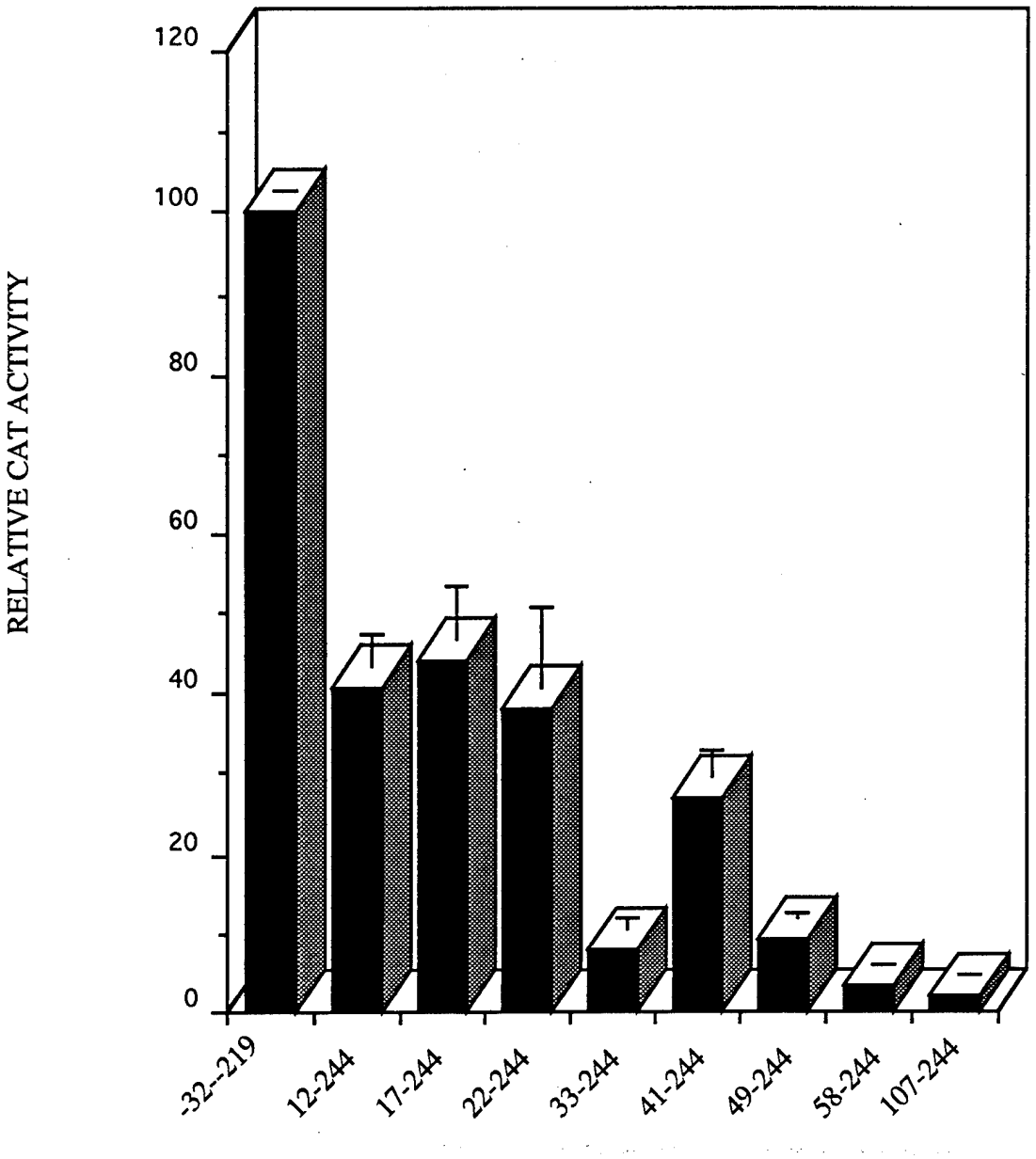


Figure 4. Transcriptional activation properties of the GAL4-v-Myc 5' (244) deletions. Relative CAT activity is presented as a percentage of the positive control, GAL4 v-Myc (-32--219). Each value represents the average of at least six independent measurements.

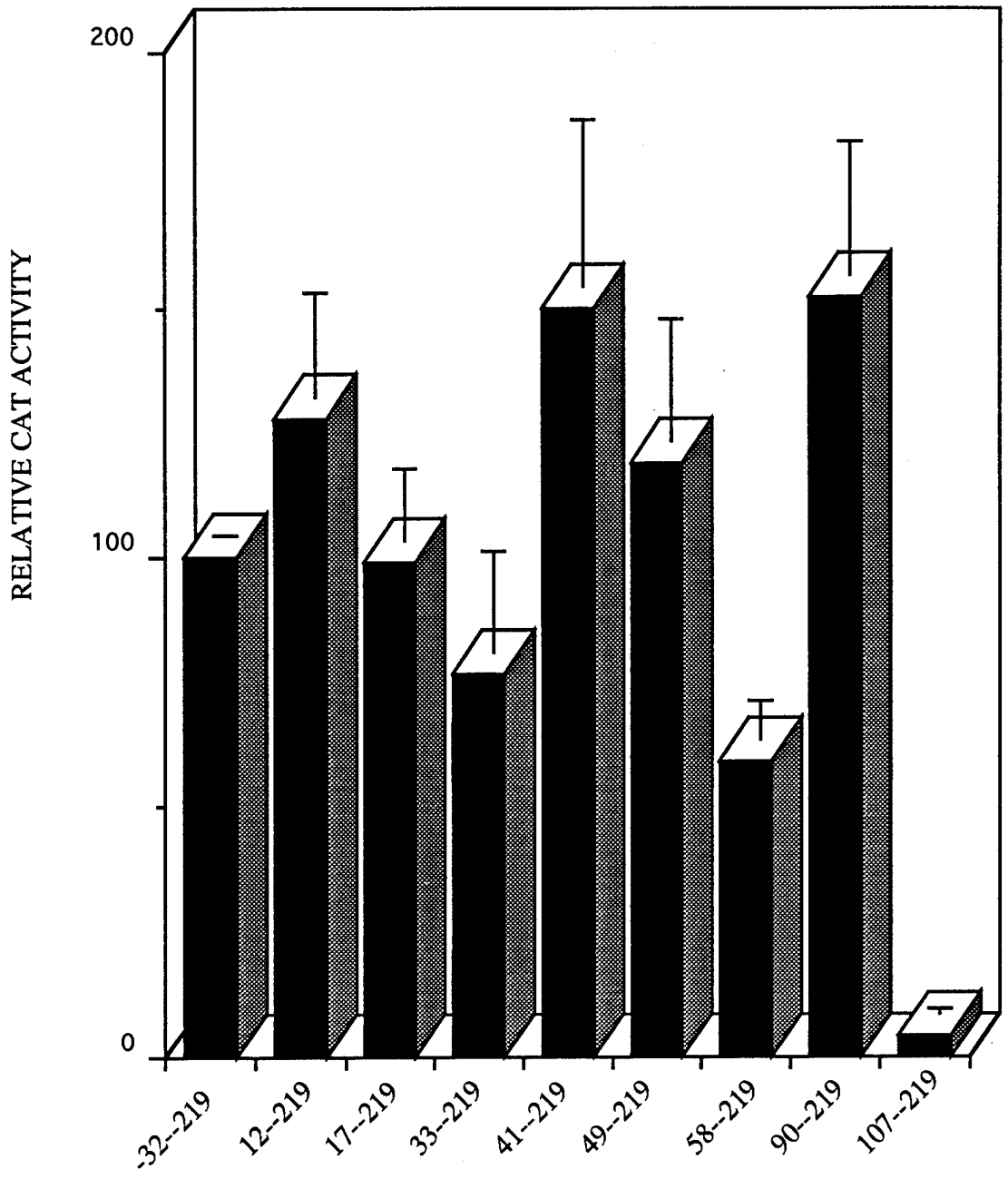


Figure 5. Transcriptional activation properties of the GAL4-v-Myc 5' (219) deletions. Relative CAT activity is presented as a percentage of the positive control, GAL4 v-Myc (-32--219). Each value represents the average of at least four independent measurements.

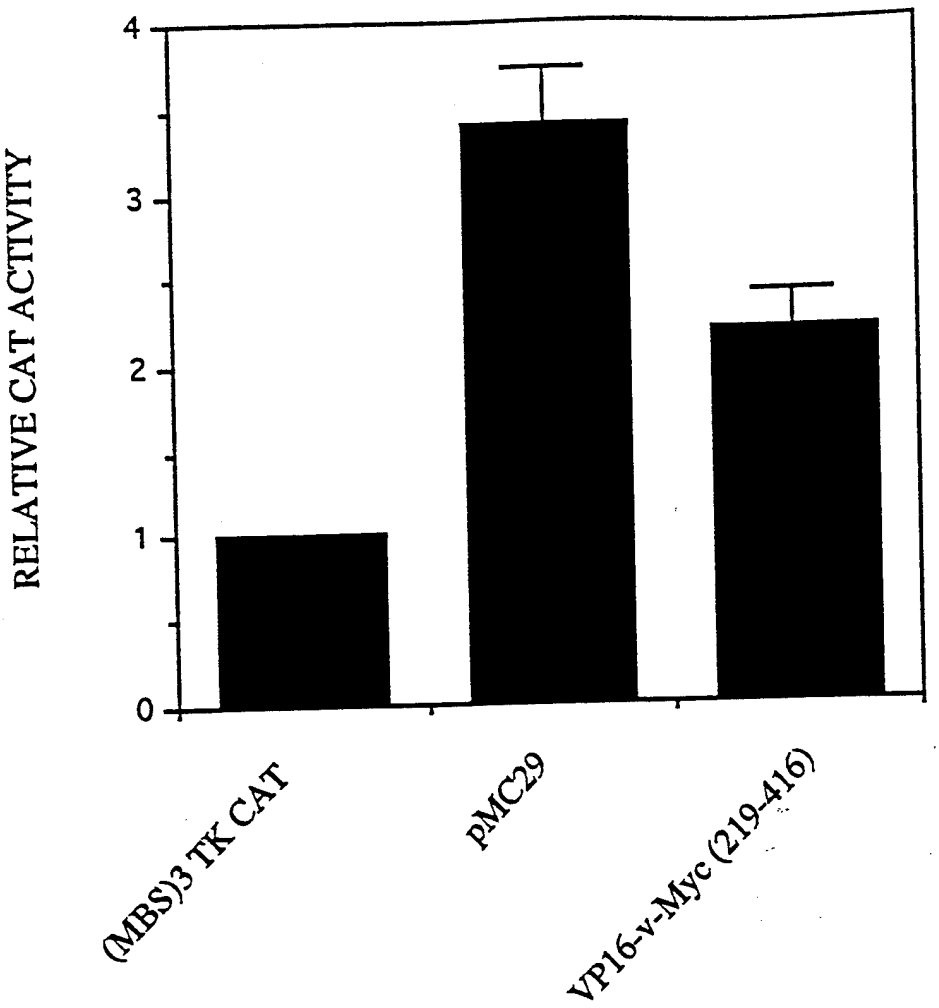


Figure 6. Transcriptional activation proterties of full length v-Myc (pMC29) and the VP16 v-Myc fusion. Relative CAT activity is expressed as fold increase over the reporter (MBS)3 TK CAT.

Construct	Efficiency of Focus Formation
ras	47%
v-myc	0%
ras + v-*myc*	100%
VP16-Myc	0%
ras + VP16-Myc	35%

Figure 7. Efficiency of focus formation of full length v-Myc (pMC29) and the VP16 v-Myc (219--416) fusion. The number of foci formed by cooperation of H-*ras* and pMC29 is set at 100%.